## REMARKS

### I. Introduction

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

Claim 1-2, 9, 10, and 13-28 are withdrawn.

Claims 3-4 and 11-12 are currently being amended.

The exemplary support for amended claims 3-4 and 11-12 may be found in the specification on page 4, line 35 through page 5, line 2; page 5, lines 30-34. Additional support may be found on page 14, lines 12-21 and page 17, lines 6-12.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

Upon entry of this Amendment, claims 1-28 will remain pending in the application, with claims 1, 2, 9, 10 and 13-28 withdrawn from consideration as a result of a restriction requirement and claims 3-8, 11, and 12 ready to be examined.

Because the foregoing amendments do not introduce new matter, entry thereof by the Examiner is respectfully requested.

### II. Response to Issues Raised by Examiner in Outstanding Office Action

#### a. Claim Rejections - 35 U.S.C. § 101

Claims 3-8, 11 and 12 are rejected by the Examiner under 35 U.S.C. § 101 as allegedly lacking patentable utility. The Examiner asserts that the specification does not support credible, substantial, or specific utility. Applicants respectfully disagree with the Examiner's findings and respectfully request reconsideration and withdrawal of the rejection.

MPEP § 2107(II) states that "an applicant need only provide one credible assertion of specific and substantial utility for each claimed invention." Additionally, an applicant needs to "establish a probative relation between the submitted evidence and the originally disclosed properties of the claimed invention." On the basis of the above recited MPEP sections, the original specification and the attached publication, applicants argue that the subject matter of the present application possesses specific and substantial utility as required under 35 U.S.C. § 101.

The specification in Table 3 teaches that SEQ ID NO.: 72 is associated with cancer and is expressed in reproductive tissues. Additionally, the specification on page 23 lines 16 through 19, describes that the invention covers GTP-binding associated proteins that may be used for diagnosis, treatment, or prevention of immune system, reproductive, nervous system, and cell signaling disorders, and cell proliferation disorders including cancer. More specifically, the specification on page 36 line 26 through page 38, line 18 and on page 49, line 9 through page 52, line 9 identifies the utility of a GBAP protein, particularly the claimed sequences, in treating and diagnosing, respectably, cancer, ovarian cancer, testicular cancer, as well as other forms of cancer.

Furthermore, applicants attach a publication disclosing a sequence of a novel GTPase protein, RAB3IP, which has 100 % similarity with SEQ ID NO:6, the peptide encoded by claimed SEQ ID NO:72. This article, Diederik R.H. de Bruijn et al., "The Cancer-Related Protein SSX2 Interacts With the Human Homologue of Ras-like GTPase Interactor, RAB3IP, and a Novel Nuclear Protein, SSX2IP," *Genes Chromosomes & Cancer*, 34:285-298 (2002), (Exhibit A), identifies RAB3IP as the human homolog of an interactor of a GTPase protein, which interacts with SSX2 and plays a role in the mechanisms underlying cellular growth, specifically malignant cellular growth. This article teaches that RAB3IP is typically present in the cytoplasm of normal cells, but in malignant testicular cells has been found to localize in the nucleus. The expression pattern taught in this article supports the use of SEQ ID NO:72, encoding the polypeptide of SEQ ID NO:6, in diagnosing and/or treating cancer. In light of this identified use for the claimed nucleotide sequence encoding the polypeptide of SEQ ID

NO:6, applicants argue that the subject matter of the claimed invention discloses at least one credible assertion of substantial utility.

Additionally, on page 8 of the July 14, 2004 Office Action, the examiner alleges that the specification does not support a specific utility for the claimed invention. Applicants argue that the specification and the publication by Diederik R.H. de Bruijn et al. article, assert a specific utility for SEQ ID NO: 72. Specifically, applicants argue that the attached publication, on page 296, teaches that RAB3IP interacts with SSX2 proteins in the nucleus of malignant testicular cells. The specification, on page 37, line 13 and page 50, line 35, teaches that the claimed nucleotide sequences encoding GTPase proteins have utility in treating and/or diagnosing, respectably, cancer of the testis. Therefore, because both substantial utility and specific utility have been established in the specification and the enclosed publication, applicants argue that the requirements of 35 U.S.C. § 101 are satisfied and that this rejection, should be withdrawn and the present claims allowed.

**b. Claim Rejections - 35 U.S.C. § 112, First Paragraph**

(i). Written Description

Claims 3, 6-8, 11 and 12 are rejected by the Examiner under 35 U.S.C. § 112, first paragraph for lack of written description. Applicants respectfully request reconsideration and withdrawal of the rejection. The Examiner asserts that no common elements or attributes of the claimed sequences are disclosed.

As set forth in *Fiers v. Revel*, 984 F.2d 1164, 1171 (Fed. Cir. 1993), an adequate written description of DNA "requires a precise definition, such as by structure, formula, chemical name, or physical properties." Applicants have amended claim 3 to cover an amino acid sequence having at least 95% sequence identity to the full length sequence of SEQ ID NO.:6 and having GTP-binding associated protein (GBAP) activity. Therefore, the genus of the present invention covers nucleotide sequences that both share 95% identity to the full length amino acid sequence of SEQ ID NO:6 and maintain GTP-binding function. Additionally, applicants amended claim 11 to cover a polynucleotide "having at least 90% sequence identity to a full length polynucleotide sequence" and claim 12 to cover a

polynucleotide having "at least 750 contiguous nucleotides of SEQ ID NO:72." Therefore, the genus of the present invention also encompasses polynucleotides containing 750 nucleotides or more that maintain at least 90% sequence identity to SEQ ID NO.:72. Applicants believe that the claims clearly define the polynucleotide genus of the present invention.

Further, the present specification identifies the structure of the claimed sequences, teaching the entire amino acid and nucleotide sequences for SEQ ID NO:6 and SEQ ID NO:72, respectively, in the disclosed sequence listing. Additionally, Table 3 provides the tissue-specific expression patterns of SEQ ID NO: 72 and Table 2 teaches the potential phosphorylation and glycosylation sites for the polypeptide of SEQ ID NO.:6. Table 2 also identifies the polypeptide of SEQ ID NO:6's signature sequence to be a signal peptide, M1-A57. Further, Table 1 provides fragments of SEQ ID NO:6 that may be used to assemble full length sequences for encoding the polypeptide.

In light of this description, applicants argue that the specification's description of the above described elements and attributes of the claimed sequences is adequate and shows applicant possession of the invention. Therefore, applicants believe that this rejection should be withdrawn and the claims allowed because the written description requirement of 35 USC §112, first paragraph has been satisfied.

(ii). Enablement

Claims 3, 6-8, 11 and 12 are rejected by the Examiner under 35 U.S.C. § 112, first paragraph for lack of enablement. Applicants respectfully request reconsideration and withdrawal of the rejection.

The examiner considers the factors set forth in *In re Wands*, 8 USPQ2d 1400 (CA FC 1988), but the focal point of his application of these factors is the inventions' alleged lack of utility. However, as set forth above, the present application does provide specific and substantial utility for the claimed inventions, i.e. the treatment and diagnosis of cancer, specifically, cancer of the reproductive tissues, more specifically, cancer of the testis. Additionally, applicants have amended the claim 3 to cover an amino acid sequence having at

least 95% sequence identity to the full length sequence of SEQ ID NO.:6 and having GTP-binding associated protein (GBAP) activity. Applicants have also amended claim 11 to cover a polynucleotide sequence maintaining a sequence identity of at least 90% to SEQ ID NO.:72 and claim 12 containing a polynucleotide of 750 nucleotides or greater. Regardless, applicants will address each of these *Wand* factors individually.

First, the examiner believes that the nature of the art is unpredictable and that the specification does not support the breadth of the claims. The nature of the art is not unpredictable because the specification provides specific and substantial utility for SEQ ID NO:72. Specifically, the specification teaches SEQ ID NO.:72 utility in diagnosing cancer of the testis. As explained above, this utility is also supported in the scientific literature. See Diederik R.H. de Bruijn et al.. Therefore, the polynucleotides utility in indicating cancer of the testis identifies the nucleotides function in a specific tissue. Therefore, the applicants respectfully disagree with the examiner and find that because the function of the nucleotide sequence has been identified, the nature of the invention is not unpredictable.

Further, applicants believe that the specification does support the breadth of the claims. Applicants amended the claims to specifically comprise "an amino acid sequence having at least 95% sequence identity to the full length sequence of SEQ ID NO.:6 and having GTP-binding associated protein (GBAP) activity," "a polynucleotide sequence having at least 90% sequence identity to a full length polynucleotide sequence" or "a polynucleotide comprising at least 750 contiguous nucleotides. As set forth above the amended claims are supported by the specification and clearly define the parameters of the present invention. Therefore, because the breadth of the claims is supported by the specification and that the nature of the invention is not unpredictable, applicants believe that this factor weighs in favor of the claims of the present invention being enabled.

Second, the examiner believes that one of skill in the art using SEQ ID NO:72 would obtain unpredictable results. Applicants respectfully disagree. Given that the skill in the art is high, as conceded by the examiner on page 16 of the pending Office Action, and based on the teachings of the specification, specifically Table 3, one of skill in the art would be able to predict that this polynucleotide would be useful for identifying cancer in reproductive tissues,

cardiovascular tissues, and gastrointestinal tissues. Therefore, one of skill in the art using SEQ ID NO:72 in the creation of transgenic animals or in gene therapy would be able to predict their results.

Third, the examiner believes that the quantity of experimentation would be high because function of nucleic acids is variable. However, as stated above the claims have been amended to specify that SEQ ID NO:72 and its fragments have the function of a GTP-binding associated proteins and the polypeptide maintains 95% identity to the full length sequence amino acid sequence of SEQ ID NO:6 or polynucleotide that maintains 90% sequence identity to SEQ ID NO.:72 or a polynucleotide that contains at least 750 nucleotides of SEQ ID NO.:72. Further, also as explained above, the utility of SEQ ID NO:72, i.e. its use in the diagnosis and/or treatment of cancer, has been described in the specification and supported by the scientific literature. Therefore, applicants do not believe that the quantity of experimentation for the claimed invention is high.

Finally, the examiner believes that the guidance presented by the specification is insufficient and determined that no working examples were provided. Applicants argue that the level of skill in the art is high and given the description and utility taught by the specification, one of skill in the art would be able to use the sequence of the present invention. Additionally, applicants remind the Examiner that applicants are not limited by their enumerated examples.

Furthermore, applicants argue that one of skill in the art would be able to make the sequence of the present invention given the high level of skill in the art and the description provided in Tables 2 and 3 of the specification. In particular, Table 2 describes the potential phosphorylation and glycosylation sites for the polypeptide of SEQ ID NO.:6 and identifies the polypeptide of SEQ ID NO:6's signature sequence to be a signal peptide, M1-A57. Additionally, Table 1 provides fragments of SEQ ID NO:6 that may be used to assemble full length sequences for encoding the polypeptide. Combining the structural information provided in these tables with the claimed sequences for SEQ ID NO:6 and SEQ ID NO:72, applicants believe that one of skill in the art would be able to make the claimed nucleotide

sequences encoding SEQ ID NO:6 or a polypeptide having at least 95% sequence identity to full length SEQ ID NO:6 and having GTP-binding associated protein activity.

In conclusion, after balancing all of the above factors, applicants believe that undue experimentation would not be required by one of skill in the art to practice the claimed invention. Therefore, applicants request that this rejection be withdrawn and the claims allowed.

**c. Claim Rejections - 35 U.S.C. § 102**

(i) Rejection as being anticipated by Brondyk et al.

Claims 3 and 6-8 are rejected by the Examiner under 35 U.S.C. § 102 as being anticipated by Brondyk et al.. Applicants respectfully request reconsideration and withdrawal of the rejection.

First, the Examiner asserts that Brondyk et al. teaches the expression of Rabin3 and its fragments as a GST-fusion protein. Applicants argue that amended claim 3 covers a polynucleotide encoding a polypeptide having at least 95% identity to the full-length sequence of SEQ ID NO:6 and GTP-binding associated protein activity. Brondyk et al. teaches a peptide that is 90% similar to SEQ ID NO.:6. See Polypeptide Sequence Comparison (Exhibit B). Therefore, because Brondyk et al. teaches a peptide that is only 90% similar to SEQ ID NO.:6, this publication does not anticipate the claimed invention.

Additionally, amended claims 11 and 12 cover a polynucleotide having at least 90% sequence identity to SEQ ID NO.:72 and a polynucleotide having at least 750 contiguous nucleotides of SEQ ID NO.:72, respectively. Brondyk et al. teaches a polynucleotide sequence, Accession No. U19181, that has a sequence identity of 83% to SEQ ID NO.:72. See Polynucleotide Sequence Comparison (Exhibit C). Further, as shown in the attached sequence comparison, Brondyk et al., does not disclose a fragment of U19181 containing 750 contiguous nucleotides of SEQ ID NO.:72. Therefore, because Brondyk discloses a nucleotide sequence that has a sequence identity of only 83% with SEQ ID NO.:72 and it

does not teach a nucleotide sequence containing at least 750 contiguous nucleotides of SEQ DI NO.:72, it cannot anticipate the present invention and this rejection should be withdrawn.

(ii) Rejection as being anticipated by Accession No. AA846576 (March 4, 1998)

Separately, claims 3 and 12 are rejected by the Examiner under 35 U.S.C. § 102 as being anticipated by Accession No. AA846576 (March 4, 1998). Applicant respectfully disagree with the examiner. As previously explained, amended claim 3 specifies that there must be at least 95% identity to full-length SEQ ID NO: 6 and Accession No: AA846576 is only 90% identical to the claimed invention. Additionally, amended claim 12 covers a polynucleotide comprising 750 contiguous nucleotides of SEQ ID NO.:72. In contrast Accession NO.: AA846576 only contains 629 contiguous nucleotides. Therefore, because Accession No: AA846576 is not 95% identical to the claimed sequence and because Accession No: AA846576 only contains 629 contiguous nucleotides, Accession No: AA846576 can not anticipate the present invention and this rejection should be withdrawn.

## CONCLUSION

The present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant(s) hereby petition(s) for such extension under 37 C.F.R. § 1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

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GENES, CHROMOSOMES & CANCER 34:285–298 (2002)

# The Cancer-Related Protein SSX2 Interacts With the Human Homologue of a Ras-like GTPase Interactor, RAB3IP, and a Novel Nuclear Protein, SSX2IP

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The *SSX* gene family is composed of at least five functional and highly homologous members, *SSX1* to *SSX5*, that are normally expressed in only the testis and thyroid. *SSX1*, *SSX2*, or *SSX4* may be fused to the *SYT* gene as a result of the t(X;18) translocation in synovial sarcoma. In addition, the *SSX1*, *SSX2*, *SSX4*, and *SSX5* genes were found to be aberrantly expressed in several other malignancies, including melanoma. The SSX proteins are localized in the nucleus and are diffusely distributed. In addition, they may be included in polycomb-group nuclear bodies. Other studies have indicated that the SSX proteins may act as transcriptional repressors. As a first step toward the elucidation of the cellular signaling networks in which the SSX proteins may act, we used the yeast two-hybrid system to identify SSX2-interacting proteins. By doing so, two novel human proteins were detected: RAB3IP, the human homolog of an interactor of the Ras-like GTPase Rab3A; and a novel protein, SSX2IP. RAB3IP did not interact with either SSX1, SSX3, or SSX4 in the yeast two-hybrid system, whereas SSX2IP interacted with SSX3 but not with either SSX1 or SSX4. Further analysis of deletion mutants showed that both RAB3IP and SSX2IP interact with the N-terminal moiety of the SSX2 protein. Immunofluorescence analyses of transfected cells revealed that the RAB3IP protein is normally localized in the cytoplasm. However, coexpression of both RAB3IP and SSX2 led to colocalization of both proteins in the nucleus. Likewise, the SSX2IP protein was found to be colocalizing with SSX2 in the nucleus. By performing glutathione-S-transferase pull-down assays, we found that both RAB3IP and SSX2IP interact directly with SSX2 in vitro. These newly observed protein/protein interactions may have important implications for the mechanisms underlying normal and malignant cellular growth. 

## INTRODUCTION

The *SSX1* and *SSX2* genes were initially identified as fusion partners of the *SYT* gene in synovial sarcomas carrying t(X;18)(p11;q11) translocations (Clark et al., 1994; Crew et al., 1995; de Leeuw et al., 1995). Typically, t(X;18) translocations result in fusion of the 78 most C-terminal amino acids of SSX1 or SSX2 to the major body of the SYT protein, replacing its 8 most C-terminal amino acids. Rare variants have also been reported, involving more N-terminally located breakpoints in the SYT and/or SSX proteins (Crew et al., 1995; de Leeuw et al., 1995; Fligman et al., 1995; Safar et al., 1998; dos Santos et al., 2001). By screening a human testis cDNA library with an *SSX* probe, de Leeuw et al. (1996) isolated another *SSX* gene, *SSX3*, that also maps to Xp11. Subsequently, the *SSX4* and *SSX5* genes were identified as aberrantly expressed genes in melanoma cell lines (Güre et al., 1997). In fact, these genes, as well as the *SSX1* and *SSX2* genes, were found to be aberrantly expressed in various human malignancies (Türeci et al., 1996, 1998; dos Santos et al., 2000a). In addition, the *SSX4* gene was recently found to be fused to the *SYT* gene in a single case of synovial sarcoma (Skytting et al., 1999). Several observations indicate that additional *SSX* gene family members may have remained unidentified to date. First, multiple *SSX*-hybridizing bands were revealed by Southern blot analysis (Chand et al., 1995; Güre et al., 1997). Second, incomplete *SSX*-like sequences distinct from *SSX1* to *SSX5* were identified in reverse transcriptase (RT)-PCR material from a primary human fibrosarcoma and in genomic fragments of an Xp11-derived cosmid clone (de Leeuw et al., 1996; dos Santos et al., 2001).

All *SSX* genes encode 188 amino acid–long proteins, with identities ranging from 77 to 91%. In

D. de Bruijn and N. dos Santos contributed equally to this work.

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normal tissues, expression at the RNA level has been observed only in the testis and, at low levels, thyroid (Crew et al., 1995; Fligman et al., 1995). SSX protein could be detected only in the testis, more specifically in early spermatogenic cells (dos Santos et al., 2000a). Inspection of the SSX amino acid sequences revealed a high content in charged amino acids, the presence of consensus sequences for N-glycosylation and tyrosine phosphorylation, and an acidic C-terminal tail that is highly conserved among the SSX family of proteins. Moreover, homology has been found between an N-terminal region of SSX and the *Krüppel*-associated box (KRAB) domain (Crew et al., 1995), a transcriptional repressor domain usually found in a subgroup of zinc-finger proteins (Margolin et al., 1994). Despite the apparent absence of a DNA-binding domain, the SSX proteins are capable of repressing the transcription of a reporter gene in transfected HeLa cells. This repressive activity was attributed to the conserved C-terminal domain (designated SSXRD domain) (Lim et al. 1998).

The SSX proteins are localized in the nucleus, as shown in transfected and nontransfected cells, distributed both diffusely and in speckles (Brett et al., 1997; dos Santos et al., 1997, 2000b; Soulez et al., 1999). We have found that a C-terminal domain of SSX (corresponding to the SSXRD domain) is responsible for SSX nuclear localization and SSX colocalization with mitotic chromosomes and polycomb-group (PcG) nuclear bodies (dos Santos et al., 2000b). The SSXRD domain is also present in SYT-SSX fusion proteins and appears to be responsible for colocalization of these proteins with both full-length SSX and PcG proteins (dos Santos et al., 2000b). Taken together, these findings indicate that the C-terminal tail of the SSX proteins is critical for the function of both the SSX and the SYT-SSX fusion proteins.

To shed further light on the function(s) of the SSX proteins and their fusion products and to identify the cellular signaling networks in which these proteins operate, we used the yeast two-hybrid system to search for interacting proteins. A human testis cDNA library was used for screening and, as a result, two candidate interactors were found. One of these is the human homolog of the rat *Rabin3* gene; the other is a novel nuclear protein that we designated SSX2IP.

## MATERIALS AND METHODS

### Cloning Procedures and Sequence Analysis

The full-length *SSX2* cDNA was cloned in-frame with the GAL4 DNA-binding domain into the pBD-GAL4 Cam vector (Stratagene, La Jolla, CA). The SSX2(Δ1–24) deletion mutant was subcloned from pCATCH-SSX2(Δ1–24) (dos Santos et al., 1997) into pBD-T3C (adapted from the pBD-GAL4-Cam vector) (de Bruijn et al., 2001a). The SSX2(Δ1–154) and SSX2(Δ155–188) deletion mutants were subcloned from the pT7T3-SSX2 vector (dos Santos et al., 1997) into the pBD-T3C and pBD-T2C vectors, respectively, using a *Bgl*II restriction site located in the SSX2 open reading frame (ORF). The pBD-SSX2(Δ116–188) deletion mutant was generated using the Erase-a-Base System (Promega, Madison, WI). The SSX2(Δ81–188) deletion mutant was obtained by PCR amplification using primers SSX-start (5′-GTGCCATGAACGGAGACGAC-3′) and SSX2-80-REV (5′-CCTGGAAGTCTTCGGCCC-3′) and cloned into the pBD-T1CA vector (adapted from the pBD-GAL4-Cam vector) (de Bruijn et al., 2001a). Similarly, the SSX2(Δ44–188) deletion mutant was amplified by PCR using primers SSX-start and SSX2–43-REV (5′-CGAGGCTTTCATCTTTTCCCACTC-3′) and cloned into the pBD-T1CA vector.

The full-length *RAB3IPα* cDNA was amplified by PCR using primers HRN3-AF (5′-ATGGCTAATGATCCCTTGGAAG-3′) and HRN3-ER (5′-GAGTTCAGGCATGGTCCCAC-3′) and cloned into the pGEM-3Zf (Promega) and pEGFP-C1 (Clontech, Palo Alto, CA) vectors. The RAB3IPα (Δ193–460) deletion mutant was amplified by PCR using primers HRN3-AF and HRN3-BR (5′-TCCTGTCCAAGTTGATCTCGC-3′) and cloned in-frame into the pGAD10-T3CA vector (adapted from the pGAD10 vector) (de Bruijn et al., 2001a). The RAB3IPβ(Δ1–179) deletion mutant was amplified by PCR using primers HRN3-CF (5′-GAGAGGCTTTCAAAAGTGCGA-3′) and HRN3-ER and cloned in-frame into the pGAD10-T3C and pEGFP-C2 vectors. The RAB3IPβ(Δ1–283) deletion mutant was amplified by PCR using primers HRN3-DF (5′-TCAGTGTGATACAGCCAATT-3′) and HRN3-ER and cloned in-frame into the pGAD10-T2CA and pSuperCATCH/BF (Georgiev et al., 1996; dos Santos et al., 1997) vectors. The RAB3IPα(Δ1–136) deletion mutant was obtained from the full-length RAB3IPa construct using a *Sca*I restriction site in its ORF and cloned in-frame into the pGAD10-T1C vector.

The full-length *SSX2IP* cDNA was isolated from the testis cDNA library (see below), digested with *Xba*I, and cloned into the pGEM-3Zf vector. The SSX2IP(Δ1–121) and SSX2IP(Δ1–197) deletion mutants used in the colocalization studies were generated by PCR using the forward primers

SSX2IP-FF (5′-ACACAGAATTTGAAGCTGG-GAAG-3′) and SSX2IP-EF (5′-ATGAAGAGAA-AAGAGCGTGAATATAATAA-3′), respectively, with the reverse primer SSX2IP-CR (5′-GACTT-TATGACACACCCTGTTTCAA-3′). The resulting cDNA fragments were cloned in-frame with the FLAG tag into the pCATCH vector (Georgiev et al., 1996). The SSX2IP(Δ1–457) deletion mutant used in the yeast two-hybrid experiments was amplified by PCR using primers SSX2IP-BF (5′-AT-TCGCCTGGGATTGGAGA-3′) and SSX2IP-CR and cloned in-frame into the pGAD10 vector. All clones were fully sequenced in both orientations using vector-specific oligonucleotides, the ABI PRISM big-dye Terminator Cycle Ready Reaction kit (Perkin Elmer, Foster City, CA) and an automated sequencer (ABI 377; Applied Biosystems, Foster City, CA) essentially as described before (de Bruijn et al., 1996, 2001a,b).

### Yeast Two-Hybrid Assays

For all yeast two-hybrid experiments, a human testis cDNA library was used, which was created using the HybriZAP kit (Stratagene). Details about the construction of this library in pAD-GAL4 have been described elsewhere (de Bruijn et al., 2001a). For the yeast two-hybrid library screening, yeast strain YRG-2 was transformed with both the bait plasmid pBD-GAL4-SSX2 and the testis cDNA library using the LiCl/urea method. Subsequent selection was carried out on SD medium lacking leucine and tryptophan. Approximately $3 \times 10^5$ transformants were harvested and replated on selective medium lacking leucine, tryptophan, and histidine, and supplemented with 20 mM of 3-aminotriazole (3-AT; Sigma, St. Louis, MO). *LacZ* activity was determined for all growing colonies using a filter-lift assay according to the manufacturer's instructions. Clones were scored as positive when blue staining was visible within 30 min at 30°C and turned more intense over the next 2 hr. The cDNA inserts from positive clones were isolated by direct PCR on yeast colonies, using pAD-GAL4-specific primers: AD-for (5′-CTGTCACCTGGTTGGACG-GACCAA-3′) and pGAD-rev (5′-GTGAACTTGC-GGGGTTTTTCAG-3′). After being sequenced, the PCR-amplified cDNA inserts were cloned into an appropriate pGAD10 vector and retransformed into YRG-2, together with pBD-GAL4-SSX2, to confirm the previously found interaction. For the assessment of deletion constructs, another yeast strain (pJ69-4A; a kind gift from Dr. Philip James) was used. Positive interactions using the pJ69-4A strain result in adenine auxotrophy, in addition to histidine auxotrophy and β-galactosidase activity.

### Database Searches

To identify homologous sequences (cDNAs, ESTs, or genomic fragments), we performed BLAST searches (Altschul et al., 1997). Protein secondary structure and coiled-coil predictions were carried out using the SOPMA (Geourjon and Deléage, 1995) and COILS (Lupas et al., 1991) algorithms, respectively. The PROSITE database (Bairoch et al., 1997) was searched to identify known protein motifs, and PSORT II analysis (Nakai and Horton, 1999) was carried out to identify signal peptides. To identify homologies between RAB3IP, SSX2IP, and other known proteins, we performed FASTA (Pearson and Lipman, 1988) and SWISS-PROT (Bairoch and Apweiler, 1999) searches.

### cDNA Library Screening

XL1-Blue MRF′ *E. coli* strain cells were infected with phages containing the human testis cDNA library cloned in HybriZAP (see above). Resultant colonies were transferred to nitrocellulose filters. These filters were hybridized at 65°C overnight with 100 ng of *SSX2IP* cDNA probe, [$^{32}$P]-labeled by random priming. The hybridized filters were washed for 20 min with 1% SSC/0.1% SDS and 20 min with 0.1% SSC/0.1% SDS, at 65°C. Hybridizing signals were detected by autoradiography. Positive lambda plaques were picked, and the phage particles were released in SM buffer (100 mM NaCl; 8 mM $MgSO_4$; 50 mM Tris–HCl, pH 7.5; 0.01% gelatin). The presence of *SSX2IP* cDNA was confirmed by PCR amplification and sequencing using a 3′-end SSX2IP primer (SSX2IP-CR) and the AD-for primer. Subsequently, the longest *SSX2IP* cDNA clone was excised in vivo from the HybriZAP vector following the manufacturer's instructions (Stratagene), and the full *SSX2IP* sequence was determined.

### Fluorescence In Situ Hybridization Analysis

Fluorescence in situ hybridization (FISH) was performed on normal human lymphocyte metaphase chromosomes essentially as described previously (Suijkerbuijk et al., 1991; de Bruijn et al., 2001b). Briefly, 300 ng of biotin-14-dATP-labeled DNA probe (BAC or plasmid) was preannealed with 40-fold excess Cot-1 DNA (Life Technologies, Gaithersburg, MD) and then hybridized overnight at 37°C to pretreated slides containing normal metaphase spreads. Immunochemical detection

was performed using fluorescein isothiocyanate (FITC)-labeled avidin (Vector Laboratories, Burlingame, CA) and several successive steps with rabbit anti-FITC and mouse anti-rabbit FITC-conjugated antibodies. The chromosomal slides were analyzed using the BDS-image software package (Oncor, Gaithersburg, MD).

### Northern Blot Analysis

Fetal and adult multiple-tissue Northern blots (Clontech) were prehybridized in a solution containing 0.5 M sodium phosphate buffer, 7% SDS, 1 mM EDTA, 1% bovine serum albumin (BSA), and 100 μg/ml herring sperm DNA. Subsequently, the blots were hybridized overnight with random-primed [$^{32}$P]-labeled cDNA probes (*RAB3IP* or *SSX2IP* cDNAs) that were preassociated with hybridime in a solution containing 0.5 M sodium phosphate buffer, 7% SDS, and 1 mM EDTA, for 90 min at 65°C. The hybridized filters were washed sequentially for 30 min at 65°C with solutions containing 1 mM EDTA, 1% SDS, and decreasing concentrations (from 0.5 to 0.05 M) of sodium phosphate buffer, followed by autoradiography.

### Cell Transfections and Indirect Immunofluorescence Assays

HeLa cells were grown in DMEM medium containing 10% fetal calf serum. Eukaryotic-expression plasmids including SSX2IP and RAB3IP cDNA fragements (see above) were transiently transfected (or cotransfected with pSG8-SSX2) into HeLa cells using DOSPER liposomal reagent, according to the manufacturer's instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN). Indirect immunofluorescence assays were performed basically as described previously (dos Santos et al., 1997), using as primary antibodies the M2 mouse anti-FLAG (Sigma) and the E3AS anti-SSX (dos Santos et al., 2000a) antibodies, and using as secondary antibody the FITC-conjugated swine anti-mouse IgG (Dako, Carpinteria, CA) antibody.

### GST Pull-Down Assays

The *SSX2IP* and *RAB3IPα* full-length cDNAs were cloned downstream to the T7 promoter in pGEM-3Zf. These cDNAs were subsequently transcribed and translated using the TnT Reticulocyte Lysate System (Promega) with [$^{35}$S]Met incorporation. GST-SSX2 fusion proteins were expressed in *E. coli* and purified as described previously (dos Santos et al., 1997). About 1 μg of GST protein was incubated with a sufficientamount of [$^{35}$S]-labeled SSX2IP protein in 20 μl buffer containing 20 mM Hepes, pH 8.0; 0.1 M KCl; 0.05% NP-40; and 2 mM $MgCl_2$ for 45 min at room temperature. Subsequently, 10 μl of glutathione-Sepharose beads (Pharmacia, Piscataway, NJ) was added to the protein mixture and 400 μl buffer containing 10 mM Hepes (pH 8.0), 0.1 M KCl, 0.05% NP-40, and 2 mM $MgCl_2$ for 90 min at 4°C. The beads were washed three times with the previous buffer and then loaded onto SDS–PAGE gels. The coprecipitating proteins were revealed by autoradiography.

## RESULTS

### Identification of Two Human SSX2-Interacting Proteins

We set out to identify SSX interactors using the yeast two-hybrid interaction trap. Because the *SSX* genes are predominantly expressed in the testis, we screened a human cDNA library derived from this tissue using the full-length SSX2 protein as a bait. The testis-derived cDNAs were fused to the GAL4 transactivation domain (TAD), whereas the *SSX2* cDNA was fused to the GAL4 DNA-binding domain (DBD). Yeast transformation with the expression and reporter vectors resulted in $4 \times 10^5$ $Leu^+/Trp^+$ transformants. Of these, 50 clones were also able to grow under *HIS3* selection and 28 also showed β-galactosidase activity. Restriction analysis revealed that three different inserts were present in these clones: a 1.7-kb insert (20 clones), a 1.5-kb insert (6 clones), and a 1.1-kb insert (2 clones). Sequencing showed that the 1.7- and 1.5-kb inserts corresponded to the human homolog of the *Rattus norvegicus Rabin3* gene. The 1.1-kb insert corresponded to a novel human gene that we designated *SSX2IP* (SSX2 interacting protein). Retransformation of the different preys with the DBD-SSX2 vector confirmed the previous results, as determined by positive β-galactosidase activity scores. In contrast, cotransformation of the isolated SSX2 preys with negative controls (DBD-p53 and DBD-Lamin C) did not yield any β-galactosidase activity.

In addition to the homology to the rat *Rabin3* gene (Brondyk et al., 1995), the 1.7- and 1.5-kb inserts also showed homology to the less well characterized murine *Pat-12* gene (Yoshida et al., 1995). Because of these homologies, the human gene we isolated was designated *RAB3IP*. Sequencing of the two *RAB3IP* cDNA clones (1.7- and 1.5-kb inserts) revealed two different alternatively spliced

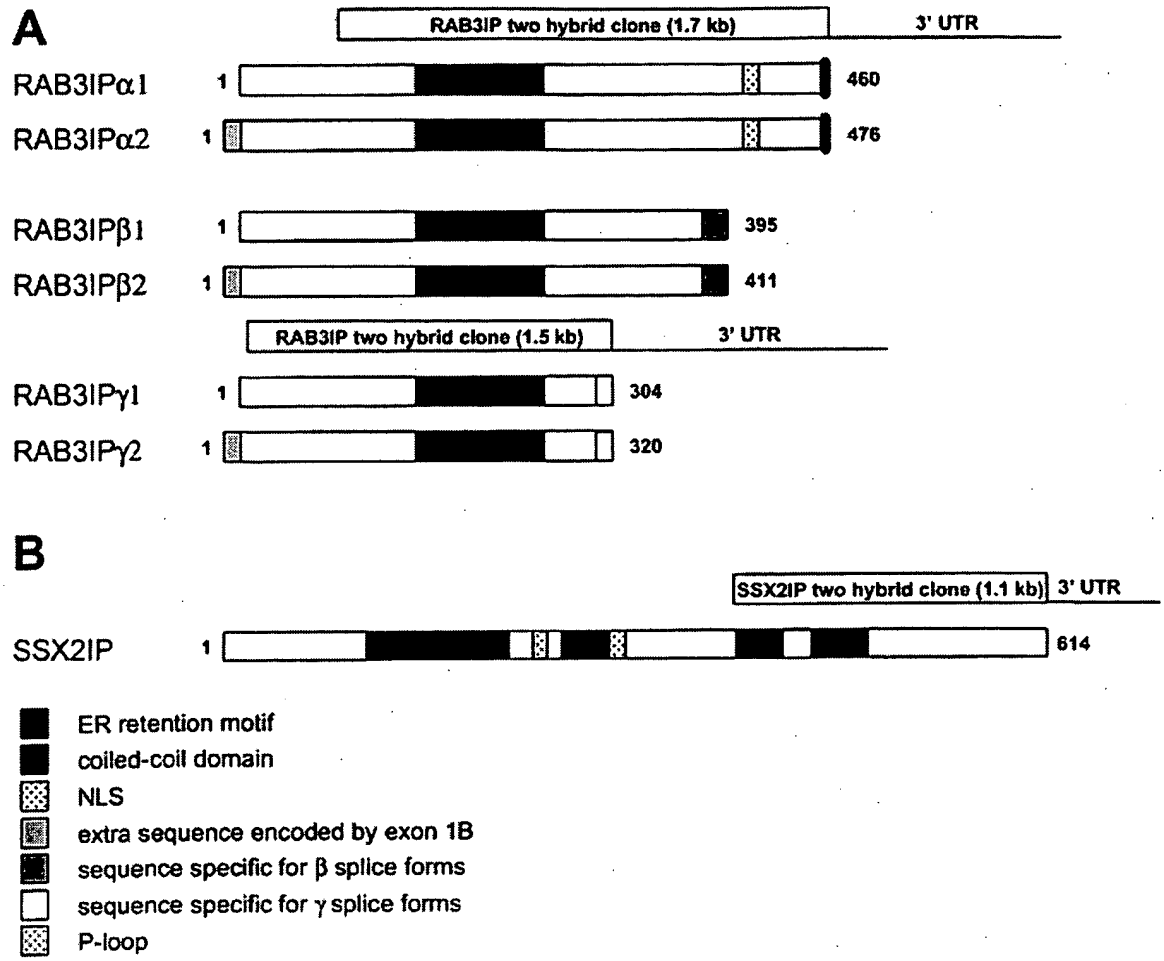


Figure 1. Schematic representation of the SSX2 protein interactors. **A:** All six RAB3IP isoforms are depicted with their putative protein domains (below). RAB3IPα1 and α2 are composed of 460 and 476 amino acids, respectively, and contain a potential coiled-coil domain, a putative NLS, and an endoplasmic reticulum (ER) retention motif. RAB3IPβ1 and -β2 are composed of 395 and 411 amino acids, respectively, and carry the same coiled-coil domain as that of the α splice forms. RAB3IPγ1 and -γ2 are composed of 304 and 320 amino acids, respectively, and carry the same coiled-coil domain as that of the α and β splice forms. The independently obtained 1.7- and 1.5-kb yeast two-hybrid clones are indicated above the α and γ splice forms. **B:** The SSX2IP protein is composed of 614 amino acids and includes four potential coiled-coil domains, a putative NLS, and a P-loop motif. The 1.1-kb yeast two-hybrid clone, carrying the last two potential coiled-coil domains, is indicated above the SSX2IP protein.

isoforms, *RAB3IPα* and *RAB3IPγ* (Fig. 1). Like the rat *Rabin3* gene, the *RAB3IPα1* isoform encodes a protein of 460 amino acids with a predicted molecular weight of 51 kDa. The human *RAB3IPα1* and rat *Rabin3* cDNAs are 84% identical, whereas the respective proteins exhibit 88% identity. The *RAB3IPγ* isoform lacks 242 nucleotides, leading to a frameshift and a premature stop. Hence, a truncated protein composed of 304 amino acids is encoded, of which the last 8 amino acids are unique to RAB3IPγ (Fig. 1).

The isolated *SSX2IP* cDNA (1.1-kb insert) included an incomplete ORF of 696 nucleotides (Fig. 1). To search for longer clones, the same testis cDNA library was screened using the 1.1-kb-long *SSX2IP* cDNA as a probe. This screening yielded several cDNAs, the longest of which included an ORF of 1,845 nucleotides encoding a peptide of 614 amino acids with a predicted molecular weight of 71 kDa. Database searches revealed that the *SSX2IP* sequence we isolated is identical to a brain-expressed 5,835-bp-long cDNA, designated KIAA0923 (accession number AB023140) (Nagase et al., 1999).

### The RAB3IP and SSX2IP Proteins Interact In Vitro With SSX2

We investigated whether the SSX2 protein interacts directly with SSX2IP and RAB3IP by performing GST pull-down assays. To this end, GST-

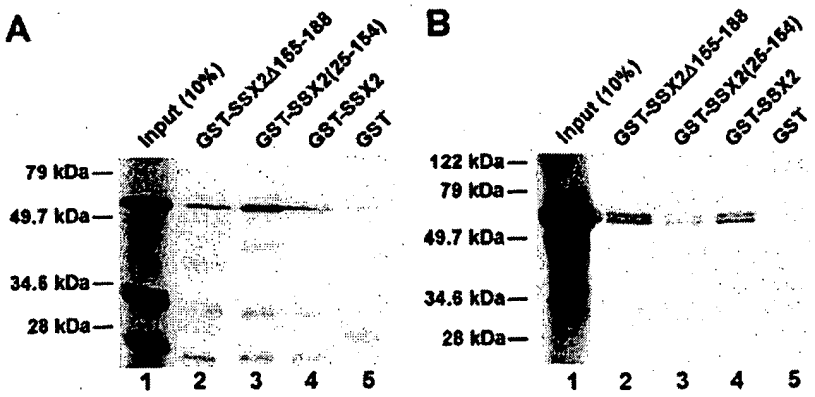


Figure 2. GST pull-down assays showing that the RAB3IP and SSX2IP proteins interact in vitro with SSX2. **A:** Radiolabeled RAB3IP protein produced by in vitro transcription and translation resulted in three protein bands of 56, 31, and 23 kDa (lane 1). The RAB3IP protein coprecipitated with GST-SSX2 (lane 4), GST-SSX2Δ155–188 (lane 2), and GST-SSX2(25–154) (lane 3), but not with GST alone (lane 5). **B:** Radiolabeled SSX2IP protein produced by in vitro transcription and translation resulted in a protein doublet of 68 and 64 kDa (lane 1). SSX2IP coprecipitated with GST-SSX2 (lane 4), GST-SSX2Δ155–188 (lane 2), and GST-SSX2(25–154) (lane 3), but not with GST alone (lane 5). Coelectrophoresed molecular weight markers are indicated in kDa.

SSX2 fusion proteins were produced in *E. coli* (dos Santos et al., 1997), whereas full-length SSX2IP and RAB3IP proteins were obtained by in vitro transcription and translation in the presence of [$^{35}$S]methionine. In vitro transcription and translation of the *RAB3IP* cDNA led to the generation of an SDS–PAGE protein band of expected size (56 kDa) and two faster migrating bands (31 and 23 kDa), most likely resulting from alternative initiation of translation or protein degradation (Fig. 2A, lane 1). After performing GST pull-down assays using different GST-SSX2 fusion proteins, we observed that RAB3IP protein coprecipitated with all of these proteins [GST-SSX2, GST-SSX2Δ155–188, and GST-SSX2(25–154)] but not with GST alone (Fig. 2A). Interestingly, under the same conditions the RAB3IP protein seemed to bind with higher affinity to the GST-SSX2(25–154) protein (Fig. 2A, lane 3), a deletion mutant that includes the minimal RAB3IP-interacting domain (see below). *SSX2IP* transcription and translation resulted in two SDS–PAGE protein bands of approximate expected size (64 and 68 kDa; Fig. 2B, lane 1). The protein doublet may, again, result from alternative initiation of translation or protein degradation. As shown in Figure 2B, the SSX2IP protein coprecipitated equally well with both full-length GST-SSX2 (lane 4) and its deletion mutant GST-SSX2Δ155–188 (lane 2). Lower amounts of SSX2IP protein were found to bind to another GST-SSX2 deletion mutant [GST-SSX2(25–154); Fig. 2B, lane 3] that lacks part of the minimal SSX2IP-interacting domain, as determined by yeast two-hybrid analysis (see below). Expectedly, no SSX2IP protein coprecipitated with GST alone. Taken together, our results indicate that the SSX2IP and RAB3IP proteins interact directly with SSX2 and that other proteins do not mediate these interactions.

### The RAB3IP and SSX2IP Proteins Interact With N-Terminal Domains of SSX2 in Yeast Cells

The GST pull-down assays indicated that RAB3IP and SSX2IP interact with the N-terminal moiety of SSX2. To determine more precisely the SSX2 domains responsible for interaction with RAB3IP and SSX2IP in yeast, we created SSX2-deletion mutants fused to the GAL4 DNA-binding domain (Fig. 3). The resulting fusion proteins were tested in yeast against the original *RAB3IP* and *SSX2IP* cDNAs fused to the TAD of GAL4. By doing so, we found that all constructs that included the most N-terminal 80 amino acids of SSX2 were able to interact with both RAB3IP and SSX2IP (Fig. 3A; doubleheaded arrows). An SSX2-deletion mutant containing amino acids 1 to 43 [DBD-SSX2(Δ44–188)] failed to interact either with RAB3IP or with SSX2IP. By testing another SSX2 construct that lacked its most N-terminal 24 amino acids [DBD-SSX2(Δ1–24)], we found that SSX2IP requires those amino acids for interaction with SSX2, whereas RAB3IP does not. Thus, both RAB3IP and SSX2IP interact with an N-terminal domain of SSX2, with RAB3IP requiring amino acids 25 to 80 and SSX2IP requiring amino acids 1 to 80. These results are in full agreement with the above GST pull-down assays and further refined the SSX2-interacting domains of SSX2IP and RAB3IP. Interestingly, the RAB3IP interaction domain almost exactly coincides with the KRAB-like domain, which spans amino acids 20 to 83. We also tested whether the SSX1, SSX3, and SSX4 proteins can interact with either RAB3IP or SSX2IP in the yeast two-hybrid system. We found that the SSX1 and SSX4 proteins do not interact with either RAB3IP or SSX2IP, whereas SSX3 interacts only with SSX2IP (not shown).

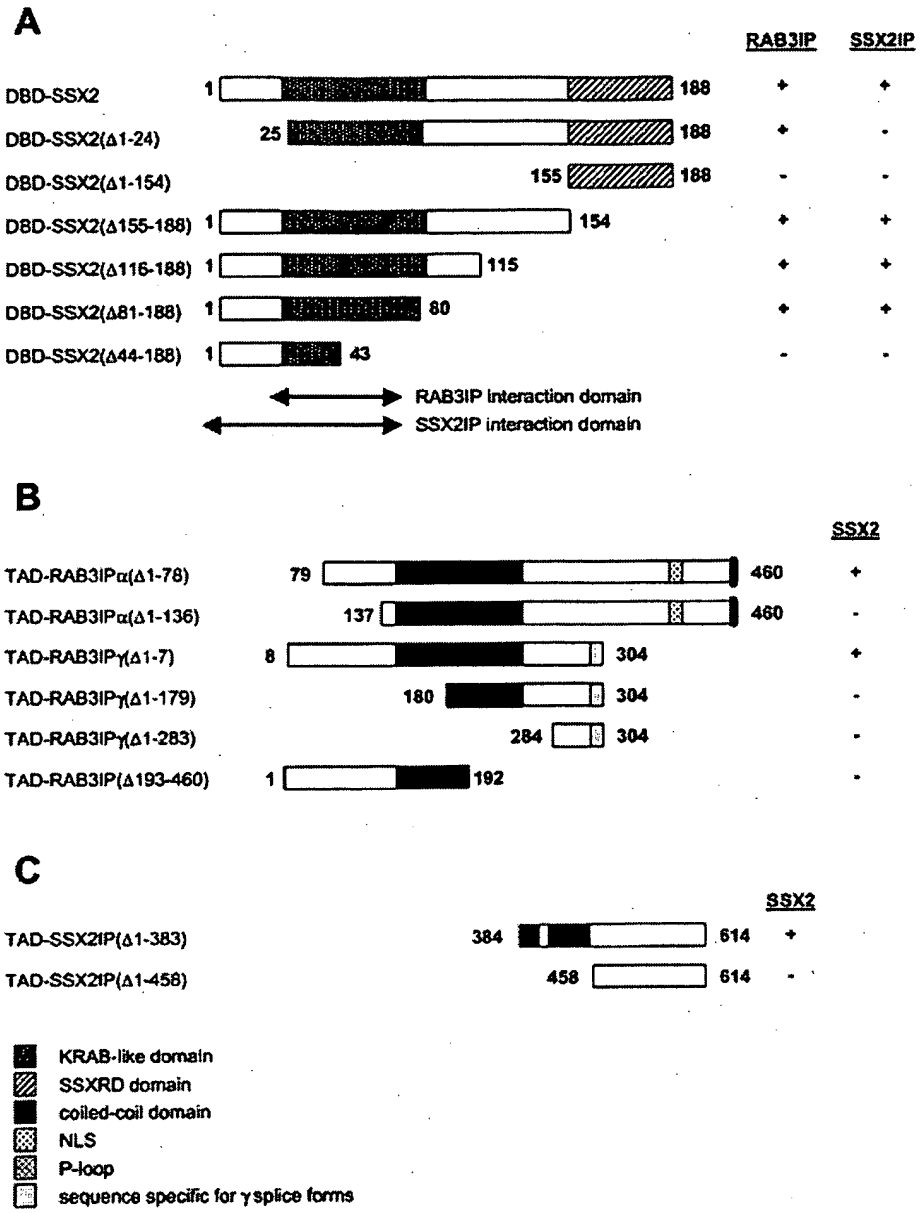


Figure 3. Determination of the domains responsible for the SSX2 interaction with RAB3IP and SSX2IP by yeast two-hybrid analysis. **A:** Coexpression of SSX2 deletion mutants fused to the DNA-binding domain of GAL4 (DBD) and RAB3IP or SSX2IP proteins fused to the transactivation domain of GAL4 (TAD). **B:** Coexpression of TAD-RAB3IP deletion mutants and DBD-SSX2. **C:** Coexpression of TAD-SSX2IP deletion mutants and DBD-SSX2. +, yeast growth; −, no yeast growth.

### Identification of the SSX2-Interacting Domains of RAB3IP and SSX2IP

To delimit the SSX2-interacting domain of RAB3IP, deletion constructs from this protein were fused to the GAL4 transactivation domain and cotransfected with full-length SSX2 in yeast. No yeast growth in selective medium was observed when SSX2 was cotransfected with both N-terminally [TAD-RAB3IPγ(Δ1–179) and TAD-RAB3IPγ(Δ1–283)] and C-terminally [TAD-RAB3IP(Δ193–460)] truncated RAB3IP constructs (Fig. 3B). These results suggest that putative SSX2-interacting domains are either interrupted or absent in all three deletion constructs or, alternatively, that both N-terminal (from position 79) and C-terminal (up to position 304) sequences are required for interaction with SSX2.

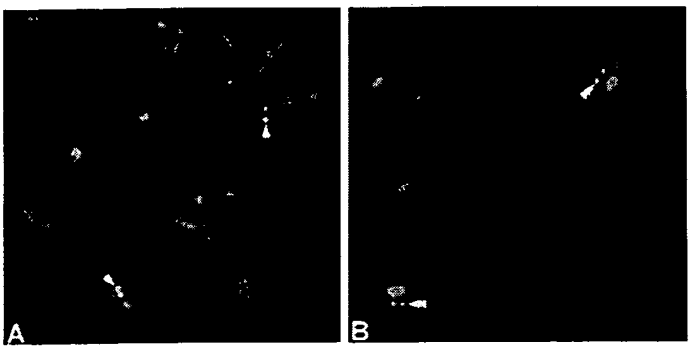


Figure 4. Determination of the chromosomal localization of the *RAB3IP* and *SSX2IP* genes by FISH. **A:** BAC clone 927F2 that includes the *RAB3IP* gene was hybridized to human metaphase spreads and yielded specific signals (arrowheads) on 12q13–14. **B:** A *SSX2IP* cDNA probe was hybridized to human metaphase spreads and yielded specific signals (double arrowheads) on 1p22.

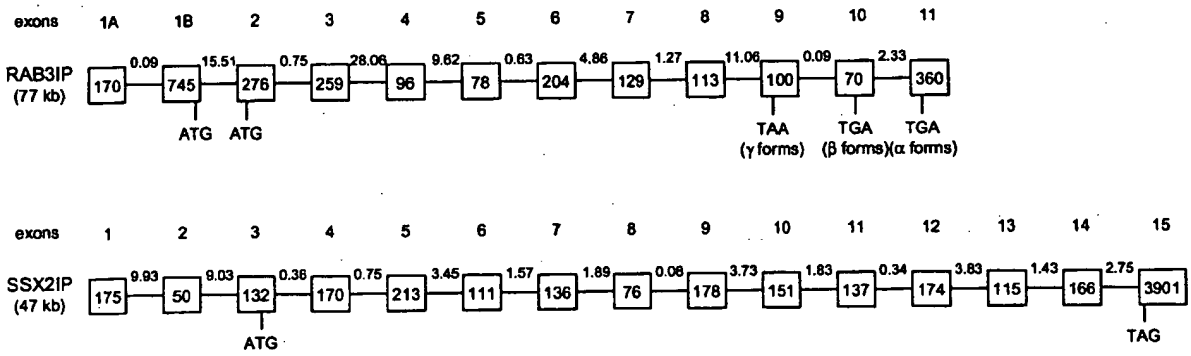


Figure 5. Schematic representation of the genomic structure of the *RAB3IP* and *SSX2IP* genes determined through comparison of their cDNA sequences with those of genomic segment NT_009540 and BAC clone RP11-45B18, respectively. All exons of both genes are shown as blocks with their sizes marked in base pairs. The sizes of the intronic sequences between the exons are marked in kilobases. Start and stop codons are indicated below the exons.

The *SSX2IP* cDNA fragment initially identified by yeast two-hybrid screening corresponded to the C-terminal end of the protein, encompassing amino acids 384 to 614 (Fig. 3C). A smaller SSX2IP fragment, composed of amino acids 458 to 614 and excluding the coiled-coil domains, was also tested for interaction with SSX2 in the yeast two-hybrid assay. No growth was observed, indicating that the SSX2-interacting domain was interrupted or deleted at position 458 (Fig. 3C).

### *RAB3IP* Is a Ubiquitously Expressed Gene Localized on Chromosome 12

An initial GenBank database search revealed that the *RAB3IP* cDNAs we isolated were identical to a human retina-derived EST (accession number W96184), located on chromosome 12, region q13–q15 (Cooper et al., 1998). To confirm this finding, we used a BAC clone that includes one *RAB3IP* exon (927F2; accession number AQ770956) as a probe in FISH analysis on normal human metaphase cells. By doing so, we found specific signals at chromosome band 12q13–14 (Fig. 4A), thus confirming and further refining the observations of Cooper et al. (1998). In addition, we found that the sequence of this locus was recently fitted into the human chromosome 12 working draft sequence (segment NT_009540). The human *RAB3IP* gene encompasses almost 77 kb and is composed of 12 exons (Fig. 5). All splice junctions conform to the gt/ag consensus (Mount, 1982). From the public expressed sequence tag (EST) data and our own data, we determined that the *RAB3IP* gene is subject to extensive alternative splicing. First, at the 5′ end, either of two alternative first exons (Figs. 1A, B, and 5) can be used. In addition, three of the internal exons (7 through 9) can be spliced alternatively. Combined, six *RAB3IP* splice products can be identified, each coding for a different isoform (Fig. 1). The α1 and α2 splice products (accession numbers AJ312896 and AJ312897, respectively) are composed of 11 exons and correspond to the 1.7-kb two-hybrid clone we previously isolated. The β1 and β2 splice products (accession numbers AJ312898 and AJ312899, respectively) lack exon 9, whereas the γ1 and γ2 splice products (accession numbers AJ312900 and AJ312901, respectively) lack exons 7 and 8 and correspond to the 1.5-kb

two-hybrid clone we previously isolated. In each case, the presence of either exon 1A or exon 1B is indicated by a number 1 or 2 in the splice form, respectively. The use of exon 1B instead of exon 1A leads to a 48-bp extension of the RAB3IP ORF because of the employment of an earlier start codon in exon 1B (Fig. 5). The absence of exon 9 in the β-splice forms leads to a frameshift and a premature stop codon in exon 10, resulting in the replacement of the final 83 amino acids of RAB3IPα by a stretch of 18 amino acids specific to the β-isoforms (Fig. 5). In the γ-splice forms, the absence of exons 7 and 8 leads to a frameshift and a premature stop codon in exon 9, resulting in the replacement of the final 164 amino acids by a stretch of 8 amino acids specific to the γ-isoforms (Fig. 5).

To determine the normal expression pattern of the *RAB3IP* gene, we performed Northern blot analysis of several tissues using a *RAB3IP* cDNA fragment as probe. By doing so, a 2.1-kb mRNA band was revealed in brain, kidney, heart, placenta, and pancreas but not in skeletal muscle and liver (data not shown). Two additional bands of 3.8 and 10.2 kb, which may result from alternative splicing, were detected in brain and kidney. From the EST data, we deduced that the *RAB3IP* gene must also be expressed in other normal tissues such as retina, fetal thymus, and B-cell germinal centers. The RAB3IPα protein has a high alpha-helical and coiled-coil potential between amino acids 150 and 250, as predicted by the SOPMA and COILS algorithms, respectively (Fig. 1). Search for potential signal peptides by PSORT II analysis revealed an endoplasmic reticulum retention motif composed of the four most C-terminal amino acids (Fig. 1). Furthermore, a potential bipartite nuclear localization signal (NLS) was recognized between amino acids 377 and 393 (Fig. 1). By searching the PROSITE database, we found several sites in RAB3IP for potential N-glycosylation, protein kinase C phosphorylation, casein kinase II phosphorylation, tyrosine kinase phosphorylation, and N-myristoylation. BLAST and FASTA searches revealed similarities between RAB3IP and several other proteins, including the *Caenorhabditis elegans* protein F54C9.11 of unknown function (Wilson et al., 1994), and the yeast transport protein Sec2, involved in vesicular transport (Nair et al., 1990). The 5′ end of this gene lacks promoter elements like the canonical CAAT and TATA boxes, but is embedded in a predicted CpG island with a GC content of over 72%. Given that such characteristics are common to housekeeping genes, the widespread *RAB3IP* expression can thus be explained.

### The RAB3IP Protein Colocalizes With SSX2 in the Nucleus

To determine the subcellular localization of the RAB3IP protein, the full-length RAB3IPα protein was fused to the enhanced green fluorescent protein (EGFP). When this fusion protein was exogenously expressed in HeLa cells, it localized predominantly in the cytoplasm in a diffuse pattern (not shown). An identical subcellular distribution was observed for two N-terminal deletion constructs of the RAB3IPβ isoform, including amino acids 180 to 304 and 284 to 304, respectively (not shown). However, when we cotransfected SSX2 and EGFP-RAB3IPα, two main staining patterns were observed. In the majority (about 90%) of doubly transfected cells, EGFP-RAB3IPα protein was localized in the cytoplasm, whereas SSX2 (detected with the anti-SSX2 monoclonal antibody E3AS) was present in the nucleus (Fig. 6a–c). Conversely, in a lower proportion of cells (about 10%) EGFP-RAB3IP colocalized with SSX2 in the nucleus, both diffusely and in nuclear speckles (Fig. 6d–i). In some cells, EGFP-RAB3IP cytoplasmic staining was also visible besides nuclear staining (Fig. 6d–f).

### *SSX2IP* Is a Novel Ubiquitously Expressed Gene Localized on Chromosome 1

BLAST searches of the GenBank and high-throughput genomic-sequence databases for *SSX2IP*-homologous genomic fragments revealed that a sequenced BAC clone (RP11-45B18; accession number AC009265) encompasses the *SSX2IP* gene. The sequence of this BAC clone has been fitted into a 1.7-megabase segment of the human chromosome 1 draft sequence (NT_004848). The *SSX2IP* gene encompasses over 46 kb and is composed of 14 exons, the first of which is untranslated (Fig. 5). All splice junctions conform to the gt/ag consensus (Mount, 1982). To investigate whether the *SSX2IP* gene has a murine ortholog, we searched the GenBank dbEST database (Boguski et al., 1993) for murine ESTs. Several overlapping ESTs were retrieved that could be assembled in a contig homologous to the 3′ end of *SSX2IP*, from codons 237 to 615. This partial *Ssx2ip* cDNA showed 82 and 85% identities to *SSX2IP* at the nucleotide and amino acid levels, respectively, indicating that the *SSX2IP* gene is evolutionary conserved. To determine the chromosomal localization of the *SSX2IP* gene, we used a cDNA clone as

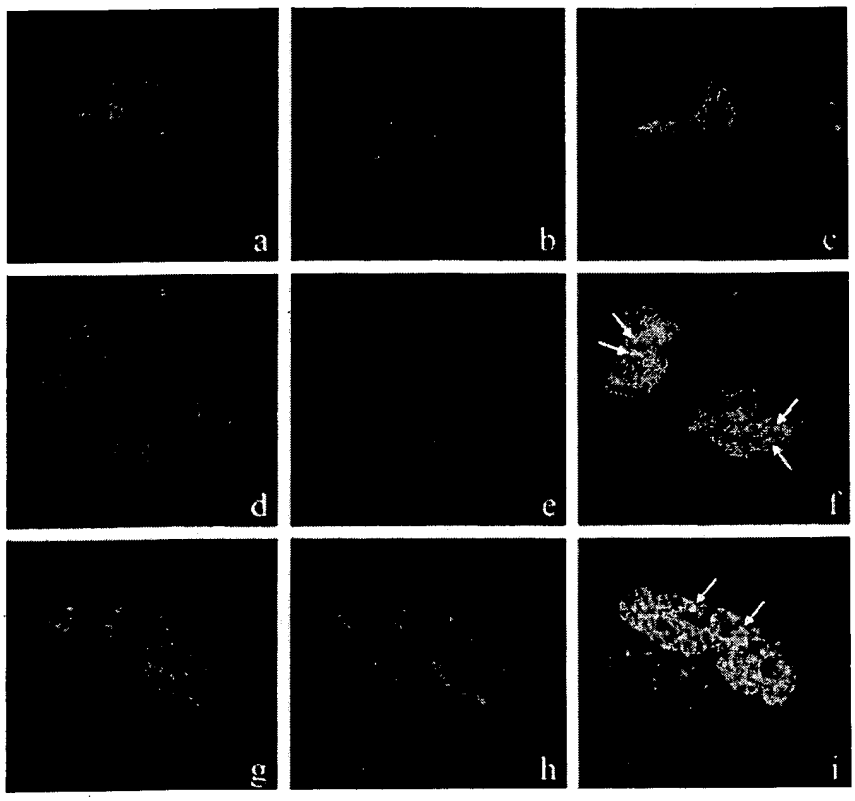


Figure 6. Subcellular localization of the EGFP-RAB3IPα and SSX2 proteins in transfected HeLa cells. **a–c:** HeLa cells expressing EGFP-RAB3IPα in the cytoplasm (a) and SSX2 in the nucleus (b). **d–f:** HeLa cells expressing EGFP-RAB3IPα in the nucleus and cytoplasm (d) and SSX2 in the nucleus (e). **g–i:** HeLa cells expressing EGFP-RAB3IPα (g) and SSX2 (h) in the nucleus. **c, f, i:** Overlays of the red and green pictures showing no colocalization (c), partial colocalization (f), and full colocalization (i). In f and i, colocalization of EGFP-RAB3IPα and SSX2 can be observed in nuclear speckles (arrows).

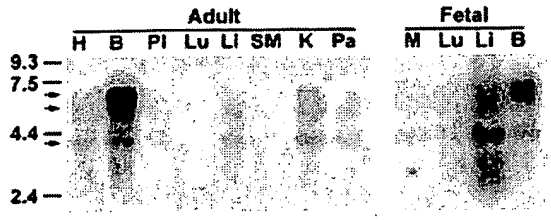


Figure 7. Northern blot analysis of the *SSX2IP* gene in adult and fetal human tissues. *SSX2IP* mRNA bands (6.7, 6.0, and 4.0 kb) are indicated by arrows. Tissues analyzed: H, heart; B, brain; Pl, placenta; Lu, lung; Li, liver; SM, skeletal muscle; K, kidney; Pa, pancreas; M, muscle. Coelectrophoresed molecular weight markers are indicated in kilobases.

probe for FISH analysis on normal human metaphase cells. By doing so, we detected specific signals only on chromosome 1, band p22 (Fig. 4B). Northern blot analysis of normal human tissues showed that the *SSX2IP* gene is ubiquitously expressed, although at markedly higher levels in the brain (Fig. 7). Three mRNA bands were detected of 4.0, 6.0, and 6.7 kb. These mRNA species may represent alternatively polyadenylated and/or alternatively spliced mRNAs. *SSX2IP* expression was also found in fetal tissues at relatively high levels in liver and, again, brain (Fig. 7). Nagase et al. (1999) found *SSX2IP* expression in all tissues tested, including different brain-derived tissues, except spleen.

Furthermore, search for *SSX2IP*-homologous human ESTs revealed several sequences isolated from additional tissues such as colon, small intestine, thyroid, prostate, and uterus. The first exon of the *SSX2IP* gene is embedded in a predicted CpG island (with a GC content of 71%) and lacks the consensual TATA-box to its 5′ end. Given that such characteristics are commonly found in housekeeping genes, the widespread *SSX2IP* expression can thus be explained. The predicted SSX2IP protein is rich in glutamic acid, leucine, and serine residues, and has a strong potential to form alpha helices and coiled-coil domains as revealed by SOPMA and COILS algorithms, respectively. Four potential coiled-coil domains were recognized at positions 117 to 229, 258 to 287, 373 to 410, and 423 to 456 (Fig. 1). A PSORT II search for signal peptides revealed a putative bipartite NLS between amino acids 285 and 300 (Fig. 1). A PROSITE search revealed sites for potential N-glycosylation, cAMP- and cGMP-dependent protein kinase phosphorylation, protein kinase C phosphorylation, casein kinase II phosphorylation, tyrosine kinase phosphor-

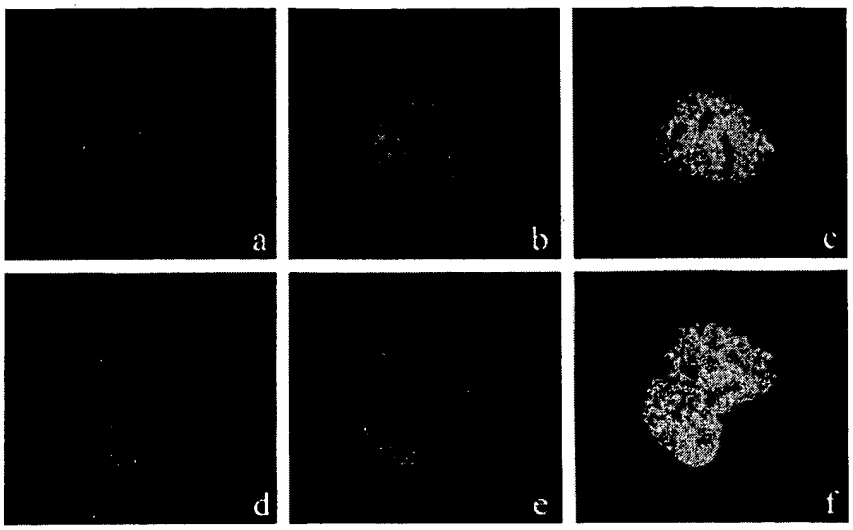


Figure 8. Subcellular localization of SSX2IP and SSX2 protein fragments in transfected HeLa cells. HeLa cells expressing FLAG-SSX2IPΔ1–121 (a) and SSX2 (b) show nuclear colocalization (c). HeLa cells expressing FLAG-SSX2IPΔ1–197 (d) and SSX2 (e) likewise show nuclear colocalization (f).

ylation, N-myristoylation, and amidation. Furthermore, the SSX2IP protein contains a putative phosphate-binding motif (P-loop) between amino acids 240 and 247, a motif that is also present in several ATP- and GTP-binding proteins. SWISS-PROT (Bairoch and Apweiler, 1999) and FASTA (Pearson and Lipman, 1988) searches revealed homology of SSX2IP with several structural proteins, most markedly with sheep trichohyalin (20% identity), an intermediate filament-associated protein of the hair follicle (Fietz et al., 1993).

### The SSX2IP Protein Colocalizes With SSX2 in the Nucleus

To assess its subcellular localization, different FLAG-tagged *SSX2IP* cDNA constructs were cotransfected with SSX2 into HeLa cells. An SSX2IP fragment encompassing amino acids 384 to 614 (FLAG-SSX2IPΔ1–383) was found to be localized exclusively in the cytoplasm (not shown). In contrast, two longer fragments including amino acids 122 to 614 (FLAG-SSX2IPΔ1–121) or amino acids 198 to 614 (FLAG-SSX2IPΔ1–197), both including the putative bipartite NLS, were localized in the nucleus. Coexpression of each of these SSX2IP constructs and SSX2 in HeLa cells led to nuclear colocalization of the SSX2IP and SSX2 proteins in a fine-speckled pattern in a proportion of the doubly transfected cells (Fig. 8a–f).

## DISCUSSION

To elucidate the function of the SSX proteins and to relate them to cellular signaling pathways, we searched for putative protein interactors using the yeast two-hybrid system. As a result we identified two interacting proteins, RAB3IP and SSX2IP. Both proteins were found to be expressed in several tissues, including the testis, the only tissue in which the SSX proteins have been detected to date (dos Santos et al., 2000a). To exclude the possibility that yeast proteins mediate the interactions of SSX2 with RAB3IP and SSX2IP, we performed GST pull-down assays and found that both interactions occurred directly in vitro. In addition, deletion-based assays revealed that both proteins interacted with N-terminal regions of SSX2 encompassing the KRAB-like domain. Because these N-terminal SSX regions are absent from the SYT-SSX fusion proteins, the observed protein/protein interactions are excluded from these proteins in synovial sarcoma cells. Furthermore, the observations that RAB3IP interacts only with SSX2 and that SSX2IP interacts only with SSX2 and SSX3 imply that, despite their high homology, the SSX proteins specifically interact with distinct proteins and thus likely perform different functions.

The *RAB3IP* gene shows a high degree of homology to the rat *Rabin3* gene. Another human sequence homologous to *Rabin3* (*RAB3IL1*, accession number AF084557) was identified and mapped to 11q12–q13 (Cooper et al., 1998). By protein-sequence alignment, we found that the rat Rabin3 protein has a higher homology to the RAB3IP protein (88% identity) than to the RAB3IL1 protein (60% identity). Interestingly, the RAB3IL1 protein is highly homologous to the rat GRAB protein (89% identity), a guanine nucleotide exchange factor for Rab3A (Luo et al., 2001). From these data, we can conclude that the RAB3IP

and RAB3IL1 genes are the bona fide human orthologs of the rat *Rabin3* and *GRAB* genes, respectively.

Initially, the rat Rabin3 protein was identified as an interactor of Rab3A (Brondyk et al., 1995), a Ras-like GTPase that is expressed in neuroendocrine cells. Rab3A is associated with secretory vesicle membranes and regulates exocytosis (Darchen et al, 1990). Rabin3 could also interact with the Rab3D but not with the Rab3C isoform. In line with the notion that the Rabin3 interaction with Rab3 proteins was specific, Brondyk et al. (1995) observed that Rabin3 did not interact in the yeast two-hybrid system with other small GTPases from the Ras superfamily (i.e., c-H-Ras, Rab2, Ran1/TC4). Furthermore, Rabin3 did not show any detectable GTPase-activating protein or nucleotide exchange activity, nor did Rabin3 have any effect on secretion processes in adrenal chromaffin cells (Brondyk et al., 1995). In contrast, the rat GRAB protein was shown to: (1) interact with Rab3A, (2) function as a guanine nucleotide exchange factor for Rab3A, and (3) regulate secretion processes in chromaffin cells (Luo et al., 2001). Taken together, these data suggest that the regulation of Rab3A function in rat relies on activation by GRAB or inactivation by Rabin3, putatively in a competitive fashion.

Here we report that RAB3IP, like Rabin3, is predominantly localized in the cytoplasm (Fig. 6a). However, the RAB3IP protein could also be found in the nucleus when coexpressed with SSX2, albeit in a low proportion of the transfected cells. Our findings show that SSX2 interacts directly with RAB3IP and may mediate the translocation of this protein to the nucleus. The *SSX2* and *RAB3IP* genes exhibit different but overlapping patterns of expression: *SSX2* is expressed only in the testis and thyroid, whereas *RAB3IP* appears to be expressed ubiquitously. The RAB3IP protein may interact with distinct proteins and perform diverse functions according to the tissue in which it is expressed. From our results, we speculate that in testicular cells the RAB3IP protein may shuttle, possibly in a cell-cycle–related fashion, between the nucleus and the cytoplasm, interacting with testis-expressed Rab3 isoforms or GTPases in the cytoplasm and with SSX2 in the nucleus. A different scenario may be at work in malignant cells in which the SSX genes are ectopically expressed (dos Santos et al., 2000a). In this setting, SSX2 expression may interfere with the normal function of RAB3IP, possibly by binding to and sequestering RAB3IP in the nucleus. Furthermore, we found that the *RAB3IP* gene is located on 12q13–14. This chromosomal region contains genes that are frequently amplified and/or rearranged in several human cancers (Mandahl et al., 1988; Forus et al., 1993). The possibility that *RAB3IP* may similarly be involved in cancer development through amplification and/or rearrangement remains to be investigated.

The function of the SSX2IP protein is, as yet, unknown. It is localized in the nucleus, as revealed by transfection assays (Fig. 8). Both the presence of four potential coiled-coil domains (Fig. 1) and the homology with several structural proteins suggest a role for SSX2IP in nuclear architecture. Colocalization with SSX2 could be observed in a fine-speckled pattern (Fig. 8), albeit in a proportion of cells. Further studies should determine the localization pattern of full-length SSX2IP and reveal to what extent it coincides with that of SSX2. Moreover, the *SSX2IP* gene was found to be localized on 1p22, a chromosomal region that is frequently altered in cancer cells. These aberrations include deletions (Knuutila et al., 1999), amplifications (Knuutila et al., 1998), and translocations (Cigudosa et al., 1999). The possibility that SSX2IP may be involved in either one of these cancer-associated genetic alterations awaits further investigation.

The SSX proteins are thought to act as transcriptional repressors (Lim et al., 1998). In addition, SSX proteins have been detected only in normal testicular cells (in particular, spermatogonia) and in malignant cells (in particular, melanomas) (dos Santos et al., 2000a). To exert their putative transcription-related function(s) in these cells, the SSX proteins very likely interact with other proteins. In the present report we demonstrate that the RAB3IP and SSX2IP proteins interact directly with SSX2. The modes of action through which the SSX2IP and RAB3IP proteins modulate the functions of SSX2 in normal spermatogonia and/or in malignant cells await further investigation.

## ACKNOWLEDGMENTS

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- ☐ 3090433CD1
- ☐ AAA67890.1

CLUSTAL W (1.83) Multiple Sequence Alignments

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Sequence format is Pearson
Sequence 1: 3090433CD1      460 aa
Sequence 2: AAA67890.1      460 aa
Start of Pairwise alignments
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Sequences (1:2) Aligned. Score:  90
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Start of Multiple Alignment
There are 1 groups
Aligning...
Group 1: Sequences:   2      Score:9520
Alignment Score 2535
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CLUSTAL W (1.83) multiple sequence alignment


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AAA67890.1      MANDPLEGFHEVNLASPTSPDLLGVCDPGTQEQTTSPSVIYRPHPSTLCSATIQANALNL
                ************************ :.******************:*.*..******::

3090433CD1      SELPTQPVYSSPRRLNCAEISSISFHVTDPAPCSTSGVTAGLTKLTTRKDNYNAEREFLQ
AAA67890.1      SDLPTQPVYSSPRHLNCAEISNISIHVPEPASSVASEVAAGLTRFTSRKDSCNAEREFLQ
                *:***********:*******.**:**.:**.. :* *:****::*:***. ********

3090433CD1      GATITEACDGSDDIFGLSTDSLSRLRSPSVLEVREKGYERLKEELAKAQRELKLKDEECE
AAA67890.1      GATVTEASAGNDDIFGLSTDSLSRLRSPSVLEVREKGYERLKEELAKAQRELKLKDEECE
                ***:***. *.**************************************************

3090433CD1      RLSKVRDQLGQELEELTASLFEEAHKMVREANIKQATAEKQLKEAQGKIDVLQAEVAALK
AAA67890.1      RLSKVRDQLGQELEELTASLFEEAHKMVREANVKQATAEKQLKEAQGKIDVLQAEVAALK
                ********************************:***************************
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3090433CD1      TLVLSSSPTSPTQEPLPGGKTPFKKGHTRNKSTSSAMSGSHQDLSVIQPIVKDCKEADLS
AAA67890.1      TLVLSSSPTSPTQEPLADGKTPFKGGHTRNKSTSSAMSGSHQDFSAIQAIVKDCREADLS
                ****************..****** ******************:*.**.*****:*****

3090433CD1      LYNEFRLWKDEPTMDRTCPFLDKIYQEDIFPCLTFSKSELASAVLEAVENNTLSIEPVGL
AAA67890.1      LYNEFRSWKDEPTMDRTCPFLDKIYQEDIFPCLTFAKSELASAVLEAVENNTLSIEPVGL
                ****** ****************************:************************

3090433CD1      QPIRFVKASAVECGGPKKCALTGQSKSCKHRIKLGDSSNYYYISPFCRYRITSVCNFFTY
AAA67890.1      QPIRFVKASAVECGGPKKCALTGQSKPCKHRIKLGDSSSYYYISPVCRYRITSVCNFFTY
                *************************.***********.******.**************

3090433CD1      IRYIQQGLVKQQDVDQMFWEVMQLRKEMSLAKLGYFKEEL
AAA67890.1      IRYIQQGLVKQQDVDQMFWEVMQLRKEMSLAKLGYFKEEL
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- ☐ 3090433CB1
- ☐ U19181.1

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 CLUSTAL W (1.83) Multiple Sequence Alignments


Sequence format is Pearson
Sequence 1: 3090433CB1     1681 bp
Sequence 2: U19181.1       1407 bp
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Aligning...
Sequences (1:2) Aligned. Score:  83
Guide tree        file created:   [baabsaqlE.dnd]
Start of Multiple Alignment
There are 1 groups
Aligning...
Group 1: Sequences:   2      Score:23969
Alignment Score 7999
CLUSTAL-Alignment file created  [baabsaqlE.aln]
CLUSTAL W (1.83) multiple sequence alignment


3090433CB1      --------GGGCAG-GGCTTTGCTATGGCTAATGATCCCTTGGAAGGCTTCCATGAAGTA
U19181.1        AGCGCTATAACTACTGAATATACTATGGCTAACGACCCCTTAGAAGGTTTCCATGAAGTG
                         *  *  * * ********** ** ***** ***** ***********

3090433CB1      AACCTTGCTTCACCTACTTCTCCGGACCTTCTTGGTGTGTATGAATCAGGAACTCAAGAG
U19181.1        AACCTCGCTTCGCCTACCTCTCCAGACCTTCTTGGTGTGTGTGACCCGGGAACCCAAGAG
                ***** ***** ***** ***** ********** ***  * ***** ******

3090433CB1      CAGACTACCTCACCAAGTGTCATCTACCGGCCACACCCTTCAGCTTTATCCTCTGTACCT
U19181.1        CAGACTACCTCCCCGAGCGTCATCTACCGGCCACACCCTTCGACGTTGTGCTCTGCCACT
                *********** ** ** ***********************  * ** * *****   **

3090433CB1      ATCCAGGCAAATGCATTAGATGTTTCTGAACTTCCTACACAACCCGTGTATTCATCCCCC
U19181.1        ATCCAGGCCAATGCCTTAAATCTTTCTGACCTTCCCACACAGCCTGTGTATTCATCCCCG
                ******** ***** *** ** ******* ***** ***** ** **************
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3090433CB1      AGACGTTTAAATTGTGCGGAAATATCTAGTATCAGCTTTCATGTTACAGACCCAGCCCCT
U19181.1        AGGCATTTAAACTGTGCAGAAATATCTAACATCAGCATTCATGTTCCAGAGCCAGCATCA
                ** * ****** ***** **********  ****** ******** **** *****  *

3090433CB1      TGCTCTACCTCTGGAGTCACAGCTGGATTAACTAAATTAACTACAAGAAAGGACAACTAT
U19181.1        TCGGTAGCCTCTGAGGTGGCAGCAGGGCTCACGAGGTTCACCTCAAGGAAGGACAGCTGC
                *      ******  **  **** **  * ** *  ** **  **** ******* **

3090433CB1      AATGCAGAGAGAGAGTTTTTACAGGGTGCTACTATAACAGAGGCTTGCGATGGCAGTGAT
U19181.1        AACGCAGAGAGGGAGTTTCTGCAGGGCGCCACCGTCACAGAGGCTTCTGCTGGCAACGAT
                ** ******** ****** * ***** ** **  * **********  * *****  ***

3090433CB1      GATATTTTTGGGTTGAGTACTGATAGTCTGTCTCGTTTACGAAGCCCATCTGTTTTGGAA
U19181.1        GACATCTTTGGGTTGAGCACGGACAGCCTGTCCCGTTTACGGAGCCCATCTGTTCTGGAA
                ** ** *********** ** ** ** ***** ******** ************ *****

3090433CB1      GTTAGAGAAAAGGGCTATGAACGATTAAAAGAAGAACTCGCAAAAGCTCAGAGGGAACTG
U19181.1        GTTAGAGAGAAGGGCTACGAAAGGTTAAAAGAAGAACTTGCAAAAGCTCAGAGGGAATTG
                ******** ******** *** * ************** ****************** **

3090433CB1      AAGTTAAAAGATGAAGAATGTGAGAGGCTTTCAAAAGTGCGAGATCAACTTGGACAGGAA
U19181.1        AAGTTAAAAGATGAAGAATGTGAGAGACTTTCGAAAGTGCGAGATCAACTTGGGCAGGAG
                ************************** ***** ******************** *****

3090433CB1      TTGGAAGAACTCACAGCTAGTCTATTTGAGGAAGCTCATAAAATGGTGAGAGAAGCAAAT
U19181.1        TTGGAAGAACTCACAGCTAGTTTATTTGAGGAAGCTCACAAGATGGTGAGAGAGGCGAAC
                ********************** ***************** ** *********** ** **

3090433CB1      ATCAAGCAGGCAACAGCAGAAAAACAGCTAAAAGAAGCACAAGGAAAAATTGATGTACTT
U19181.1        GTCAAGCAGGCAACTGCAGAAAAGCAGCTGAAGGAAGCTCAAGGGAAAATTGATGTGCTT
                 ************* ******** ***** ** ***** ***** *********** ***

3090433CB1      CAAGCTGAAGTAGCTGCATTGAAGACACTTGTATTGTCCAGTTCTCCAACATCACCTACG
U19181.1        CAAGCCGAAGTGGCTGCGCTGAAGACACTCGTACTGTCCAGTTCTCCAACATCGCCTACG
                ***** ***** *****  ********** *** ******************** ******

3090433CB1      CAGGAGCCTTTGCCAGGTGGAAAGACACCTTTTAAAAAGGGGCATACAAGAAATAAAAGC
U19181.1        CAGGAGCCTCTGGCGGATGGAAAGACGCCCTTTAAGGGGGGGCACACACGGAACAAAAGT
                ********* ** *. * ********* ** *****   ****** *** * ** *****

3090433CB1      ACAAGCAGTGCTATGAGTGGCAGTCATCAGGACCTCAGTGTGATACAGCCAATTGTAAAA
U19181.1        ACAAGCAGTGCTATGAGTGGCAGCCACCAGGACTTCAGTGCGATCCAGGCAATCGTAAAA
                ********************** ** ****** ****** *** *** **** ******

3090433CB1      GACTGCAAAGAGGCTGACTTATCCTTGTATAATGAATTCCGATTGTGGAAGGATGAGCCC
U19181.1        GACTGCAGAGAGGCCGATCTATCGCTGTATAATGAGTTCAGGTCTTGGAAGGATGAGCCC
                ******* ****** **  **** ********** *** * *  ***************

3090433CB1      ACAATGGACAGGACGTGTCCTTTCTTAGACAAAATCTACCAGGAAGATATCTTTCCATGT
U19181.1        ACAATGGACAGAACATGTCCCTTCCTAGACAAAATCTATCAGGAGGATATCTTTCCATGC
                *********** ** ***** *** ************* ***** **************

3090433CB1      TTAACATTCTCAAAAAGTGAGTTGGCTTCAGCTGTTCTGGAGGCTGTGGAAAACAATACT
U19181.1        TTAACATTCGCGAAAAGTGAGTTGGCTTCAGCTGTTCTGGAGGCCGTGGAGAACAACACT
                ********* * ********************************* ***** ***** ***
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3090433CB1      CTAAGCATTGAACCAGTGGGATTACAACCTATCCGGTTTGTGAAAGCTTCTGCAGTTGAA
U19181.1        CTAAGCATCGAGCCGGTAGGGTTGCAGCCCATCCGCTTCGTGAAGGCTTCCGCGGTGGAG
                ******** ** ** ** ** ** ** ** ***** ** ***** ***** ** ** **

3090433CB1      TGCGGAGGACCAAAAAAATGTGCTCTCACTGGCCAGAGTAAGTCCTGTAAACACAGAATT
U19181.1        TGCGGAGGGCCAAAAAAGTGTGCTCTCACTGGCCAGAGTAAGCCCTGTAAGCACAGGATT
                ******** ******** ************************ ******* ***** ***

3090433CB1      AAATTAGGGGACTCAAGCAACTATTATTATATTTCTCCTTTTTGCAGATACAGGATCACT
U19181.1        AAACTAGGGGACTCCAGCAGCTATTACTACATCTCTCCGGTTTGCAGATACCGGATCACT
                *** ********** **** ****** ** ** *****  *********** ********

3090433CB1      TCTGTATGTAACTTTTTTACATACATTCGATACATTCAGCAGGGACTCGTGAAACAGCAG
U19181.1        TCTGTATGTAACTTTTTTACATACATTCGATATATTCAGCAGGGACTTGTGAAACAGCAG
                ******************************** ************** ************

3090433CB1      GATGTTGATCAGATGTTTTGGGAGGTTATGCAGTTGAGAAAAGAGATGTCATTGGCAAAG
U19181.1        GATGTTGATCAGATGTTCTGGGAAGTTATGCAACTAAGAAAGGAGATGTCGTTGGCAAAG
                ***************** ***** ********  * ***** ******** *********

3090433CB1      CTGGGTTATTTCAAAGAGGAACTCTGATGCTCTGCGTGGGACCATGCCTGAACTCCCCGA
U19181.1        CTGGGATATTTCAAAGAAGAACTCTGA--------------------------------
                ***** *********** *********

3090433CB1      ATAACTGAAAAATGGCTGAATATTTTTATGGTTACTTGATATTTATTTCCAAGGAGTGAG
U19181.1        ------------------------------------------------------------

3090433CB1      CCTAAGACTTTTTTCCCCTTTTGCAAATTGCTCTAAGAAGTACCATGATTTCTTTTAAAC
U19181.1        ------------------------------------------------------------

3090433CB1      TGATCTATGCTGTGTTTGCTTATTCTTTAGTTGAACACACTATGAAGAATTCCAGGTGTA
U19181.1        ------------------------------------------------------------

3090433CB1      CTAGTGAATGTAATTTATAGTTGCCAAAAAAAAAAACAAACCTGAAATAAATAAATGTTAG
U19181.1        ------------------------------------------------------------

3090433CB1      ATTGAAAAAA
U19181.1        ----------
```

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